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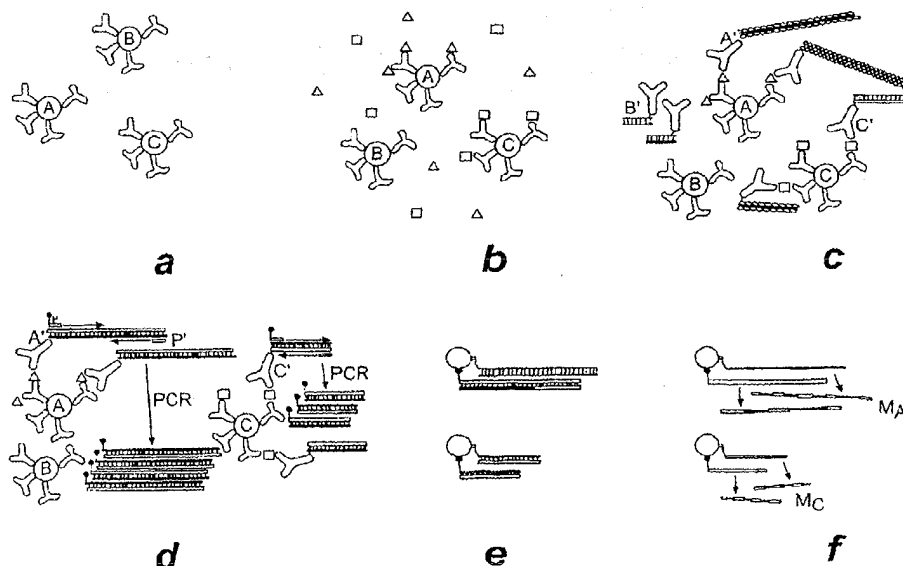
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(54) Title: A SENSITIVE, MULTIPLEXED MASS-SPECTROMETRY-BASED BIOSENSOR BASED ON IMMUNO-PCR



WO 01/84146 A2

(57) Abstract: The invention provides a method for detection of substances. The method utilizes immunological detection of a substance, followed by amplification of a nucleic acid tag bound to the antibody, and identification of the amplified tag. The detection method utilizes mass spectrometry to detect specific binding of the antibody to an antigen by detecting the presence, and identifying the mass, of the nucleic acid tag.

WO 01/84146 A2



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**A Sensitive, Multiplexed Mass-Spectrometry-Based Biosensor  
Based on Immuno-PCR**

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

This invention relates to systems for detection of substances, such as those encountered in biological systems. In particular, it relates to systems, including assays, devices, and kits, for detection of biologically relevant molecules using a combination of techniques practiced in the fields of immunology, nucleic acid amplification, and mass spectrometry.

**Description of Related Art**

The field of genomics is rapidly maturing and providing increasing information not only about genes, genome content, structure, and function, but about the proteins encoded by the analyzed genes and genomes as well (*i.e.*, proteomics). Thus, it is considered imperative to seek improvements to existing proteomics technology in order to further our understanding of proteins and their interactions with genes and other proteins to carry about the functions of living cells (ref. 1).

A major goal in proteomics research will be to develop methods and devices for sensitive, selective, rapid, and parallel monitoring of a multitude of proteins, metabolites, drugs, *etc.*, especially as a function of the state of an organism (refs. 2, 3). Achievement of this goal is hampered by, among other things, the complexity of protein structures and states of activation. For example, it is known that various post-translational modifications of proteins can have significant functional implications. Achievement of this goal is also hampered by the fact that some proteins are functionally significant (*i.e.*, have a biologically significant effect) even at the level of a few copies per cell, levels far too low to be adequately addressed with current techniques and devices. Therefore, as part of the development of methods and devices for proteomics research, ways of "amplifying" proteins will need to be developed. Unfortunately, there is no direct means for such amplification known at this time. Therefore, indirect means have been, and are being, developed.

It is considered likely that immunological techniques can assist in assaying proteomes and metabolomes that comprise a plurality of diverse targets. It is known that antibodies can be raised against proteins, peptides, metabolites, and even inorganic particles. It is also known that antibodies can be raised that are specific for a given protein, but that are sensitive to protein conformation and to the state of post-translational modification as well (ref. 4). In addition, the construction of large libraries of useful probe antibodies is now within reach (ref. 5). In general, the large probe libraries are possible due to the fact that antibodies possess both a variable (or "hypervariable") region and a constant (or "C") region. The variable region manifests binding specificity while the constant region displays antibody class specificity determinants. The C-terminus regions of the antibody heavy chains can serve as a useful "platform" onto which chemical moieties can be attached. These additional moieties can be selected so as to impart a property useful in analytical applications. One useful property is the ability to amplify.

Research and analytical chemistry involving genetic material benefit enormously from the fact that DNA segments can be amplified by the polymerase chain reaction (PCR) (ref. 6). Amplification by  $> 10^6$  with a sensitivity to as little as one copy of target DNA in a sample can be achieved. PCR products are commonly monitored by gel electrophoresis with staining or fluorescent/radioactive labelling, a slow, tedious process. Biological mass spectrometry (MS), including electrospray ionization (ESI) (ref. 7) and matrix-assisted laser desorption ionization (MALDI) MS (ref. 8), provides a powerful alternative means for analyte identification. Such forms of analysis are being applied to DNA (ref. 9), and it has even been demonstrated that MS techniques can be employed to sequence a genome (ref. 10). It is appreciated that mass measurements with sufficiently high accuracy are capable of revealing length and base composition of DNA amplified by PCR (ref. 11). Such measurements provide an enhancement in speed over analysis by gels (refs. 9, 12). As examples of applications, PCR with MALDI-MS has been demonstrated as a rapid means of assaying bacterial populations in real-life environments (ref. 13), as well as studying the genetic diversity of short tandem repeats (STR) (ref. 14).

One generic technique for indirectly "amplifying" proteins and other analytes or small molecules is enzyme-linked immunosorbant assay (ELISA), wherein the protein, analyte, or small molecule is the target of a specific antibody which has an enzyme linked to it. Upon admission of the substrate of the enzyme into the system, product is generated in a repetitive (*i.e.* amplified) fashion. Analyte is normally identified by color change, and quantified by changes in optical absorbance.

It is also known to couple immunological techniques (*e.g.*, ELISA) with MS readout (refs. 15, 16). In one method, a phosphatase is conjugated to a secondary antibody directed against a monoclonal antibody, which is specific for a molecule presented on the surface of liver cells. The phosphatase is used to dephosphorylate a substrate. Products of altered mass are then detected by MALDI-MS in the presence of primary antibody binding (ref. 15). The general scheme is disclosed in WO 98/26095 (ref. 16). In this type of system, presented antigen is effectively "amplified".

In principle, ELISA can be multiplexed. However, due to the generic broadness of spectral response, detection by adsorption/fluorescence is not considered to be promising beyond about a 10-plex methodology. That is, due to the potential interference between tags, it is not believed that more than 10 tags can be used concurrently in a single assay. Detection by MS admits a much greater degree of multiplexing, as has been suggested for ELISA (ref. 16), since MS can read out many masses simultaneously.

A relatively new area for indirectly "amplifying" proteins or other small molecules is immuno-PCR (ref. 17). In this method, instead of linking an enzyme to an antibody, a piece of DNA is linked. Upon antibody binding to an analyte, protein, or small molecule, the DNA linked to the antibody is available to be amplified by PCR. An immuno-PCR scheme that is considered to be "universal" involves: *i.* biotinylating the antibody; *ii.* biotinylating *one* of the DNA strands of the tag; and *iii.* using streptavidin to irreversibly bind the DNA tag to the secondary antibody (ref. 18). A variety of works have been published in which ELISA has been compared with immuno-PCR. Enhancement factors of  $10^4$ - $10^8$  have been claimed in favor of immuno-PCR. A triplex immuno-PCR reaction with three different DNA tags was employed to detect hTSH, hCG and  $\beta$ -Gal (refs. 19, 20).

Several U.S. and foreign patents disclose immuno-PCR techniques. For example, U.S. Patent No. 5,665,539 to Sano *et al.* discloses immuno-PCR detection of a single analyte, while U.S. Patent No. 5,985,548 to Collier *et al.* discloses a multiplex immuno-PCR technique to measure several analytes. (A more "universal" scheme for tagging antibodies with DNA is given by Zhou *et al.* (ref. 18), for example.) In addition, U.S. Patent No. 5,770,367 to Southern & Cummins discloses a "tag reagent and assay method". The method is disclosed as applicable to photo-cleavable tags and DNA sequencing.

Furthermore, Montforte *et al.* (WO 98/26095) discloses the concept of creating large libraries of compounds permitting rapid screening for a wide variety of targets simultaneously. This publication discloses a method for detecting a target molecule where the target molecule is amplified to produce an amplified target molecule. In essence, this publication discloses ELISA with MS readout; however, it does not disclose immuno-PCR.

Others have disclosed the utility of MS for characterizing DNA and the utility of using MS in conjunction with DNA as a tag. Included among these are U.S. Patent No. 5,580,733 to Levis & Romano; U.S. Patent No. 5,643,798 to Beavis & Chait; U.S. Patent No. 5,885,775 to Haff & Smirnov; U.S. Patent No. 5,985,548 to Collier *et al.*; and U.S. Patent No. 6,027,890 to Van Ness *et al.*

However, while many of the immuno-PCR techniques show promise and provide various advantages, none of them has proven to be sufficiently sensitive, selective, and rapid to be applicable for parallel monitoring of a multitude of proteins, analytes, and small molecules.

### **SUMMARY OF THE INVENTION**

The systems of the present invention provide the sensitivity, selectivity, and rapidity necessary for obtaining useful information on proteomes and metabolomes, as well as on various analytes or small molecules present in samples. The systems of the present invention utilize DNA as an amplifiable tag for antibodies. However, unlike immuno-PCR systems known in the art, the systems of the present invention identify the amplified tag, and hence the detected analyte, protein, small molecule, *etc.*, by

mass spectrometry. By employing a plurality of unique mass tags, a large number of analytes can be assayed in parallel.

In a first aspect, the invention provides a method for identifying and/or quantitating at least one molecule or compound present in a sample suspected of containing the molecule(s) or compound(s). As used herein, "molecule(s)" means any element of the periodic table, or combination of elements in any proportions or amounts. As used herein, "compound(s)" means any group of elements covalently, ionically, or otherwise linked by atomic interactions between molecules such that they form a chemically recognizable entity. "Compound(s)" includes both organic and inorganic compounds. Organic compounds are compounds that contain carbon molecules. Inorganic compounds do not contain carbon compounds. Accordingly, as used herein, molecule(s) includes compound(s).

In general, the methods of the invention comprise a) providing at least one antibody that specifically binds to a molecule or compound (antigen) of interest, wherein the antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture; b) providing a sample that is suspected of containing the molecule or compound of interest; c) combining the antibody and the sample; d) combining a second antibody, which is tagged with a nucleic acid and which is specific for the molecule or compound of interest, with the first antibody to form a second assay composition; e) separating any complexes of first antibody/antigen/second antibody from other components present in the second assay mixture; f) amplifying the nucleic acid tag bound to the second antibody in an amplifying composition; and g) detecting the presence and/or quantity of amplified nucleic acid by mass spectrometry, wherein the presence of amplified nucleic acid indicates the presence of the molecule or compound of interest in the original sample. The amount of amplified nucleic acid can be used to indicate the amount of the molecule or compound present in the original sample.

In another aspect, the invention provides a kit for practicing the method of the invention. The kit comprises some or all of the reagents and supplies for practicing the method of the invention, packaged separately or in combination in a suitable

container. As used herein, a "container" is any physical entity that is capable of containing a solid, liquid, or gas. Thus, a container can be a vial, ampoule, syringe, tube, etc.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** shows a general scheme for one embodiment of the immuno-PCR-MS method of the invention. In this embodiment, the immuno-PCR-MS method is practiced in parallel. Briefly, a) bead-immobilized antibodies (Y-shapes) A, B, and C are present in solution; b) antigens (squares, triangles) are sampled into the system (in the case shown, antigens targeted by A and C are present and bind accordingly); c) complementary antibodies A', B', and C' are sampled into the system (each unique antibody is tagged with a characteristic DNA strand); d) after uncoupled B' antibodies are washed away, PCR is carried out (DNA strands attached to A' and C' are amplified; primer P is biotinylated, primer P' is not); e) thereafter, amplified DNA is captured on streptavidin-labeled magnetic beads in a distinct orientation; f) after washing, single-stranded DNA is released from immobilized beads by thermal denaturation, aliquots acquired, and the masses  $M_A$  and  $M_C$  are determined.

**Figure 2** shows representative histograms of mass analysis of DNA tags.

**Figure 2a** shows the histogram of masses ( $m/z$  values) for 10-base single-stranded DNA centered around  $m/z \sim 3100$ .

**Figure 2b** is a detailed depiction of a portion of Figure 2a, and shows that unique mass tags can be spaced every  $\sim 1$  Da (singly-charged ions) over a broad total range, so that a single-stranded DNA of 10 bases offers some 300 tags.

**Figure 3** shows an embodiment of the invention in which nucleic acid tags are first processed by restriction endonuclease cleavage prior to detection with mass spectrometry. The processing proceeds from step a) to b) to c).

### **DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION**

When studying biopolymers constructed out of specific building blocks, like amino acids or nucleotide bases, mass information can be used for identification (refs. 11, 19, 21, 22, 23). Therefore, carefully selected biopolymers and other chemical



moieties can serve as identification "tags". The immuno-PCR methods of this invention use the mass of an amplified nucleic acid (*e.g.*, DNA) tag itself as the feature that indicates the presence and/or quantity of a molecule or compound suspected of being in a tested sample. The average molecular mass of each base (G, C, A, T) is given as follows:  $M_G=329.2089$  Da;  $M_C=289.1843$  Da;  $M_A=313.2096$  Da; and  $M_T=304.1959$  Da. By using different combinations and lengths of bases in the tag, a fine mass spacing is accessible for a very modest total number of bases.

Thus, the present invention provides a multiplexed immuno-system for analyte detection. The system uses specific antibody-antigen binding, nucleic acid amplification, and mass-spectrometric readout. The methodology by-passes inefficient current methodologies, such as gel electrophoresis.

The present invention provides a system for probing for tens to hundreds to thousands of different analytes (antigens), all at the same time, where the range of analytes probed depends only on the ability to build a suitable library of antibodies. The sensitivity, per analyte, is enhanced relative to what is normal for mass spectrometry at present. The present invention, while not incompatible with two-dimensional array formats, actually excels at probing analytes that might be present in a common three-dimensional solution phase. Therefore, absolute localization of reagents and products in a two-dimensional space is not a limiting feature, as in the systems currently widely available. Accordingly, one can probe for an endless range of parameters in the same container. Furthermore, while amenable to chips and arrays, practice of the invention is not limited thereto.

In general, the methods of the invention comprise a) providing at least one antibody that specifically binds to a molecule or compound (antigen) of interest, wherein the antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture; b) providing a sample that is suspected of containing the molecule or compound of interest; c) combining the antibody and the sample; d) combining a second antibody, which is tagged with a nucleic acid and which is specific for the molecule or compound of interest, with the first antibody to form a second assay composition; e) separating any complexes of first antibody/antigen/second antibody from other components present

in the second assay mixture; f) amplifying the nucleic acid tag bound to the second antibody in an amplifying composition; and g) detecting the presence and/or quantity of amplified nucleic acid by mass spectrometry, wherein the presence of amplified nucleic acid indicates the presence of the molecule or compound of interest in the original sample. The amount of amplified nucleic acid indicates the amount of the molecule or compound present in the original sample.

In embodiments, the invention provides a method for identifying and/or quantitating at least one molecule or compound (sometimes referred to herein generally as an antigen) present in a sample suspected of containing the molecule(s) or compound(s). In these embodiments, the method comprises: a) providing at least one antibody (referred to hereafter as the "first" antibody) that specifically binds to a molecule or compound, wherein the first antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture; b) providing a sample that is suspected of containing a molecule or compound of interest; c) combining the first antibody and the sample to form a first assay composition; d) subjecting the first assay composition to conditions under which the first antibody and molecule or compound, if present, can specifically interact, e) combining a second antibody, which is tagged with a nucleic acid and which is specific for the molecule or compound of interest, with the first antibody, which will be bound to the analyte of interest if it was present in the tested sample, to form a second assay composition; f) subjecting the second assay composition to conditions under which the second antibody and molecule or compound, if present, can specifically interact, wherein specific interaction results in formation of a complex between the first antibody, the molecule or compound, and the second (tagged) antibody; g) separating the complex from other components present in the second assay mixture; h) amplifying the nucleic acid tag bound to the second antibody in an amplifying composition; i) binding amplified nucleic acid molecules to a solid support, wherein the solid support is different than the solid support linked to the first antibody; j) separating the bound amplified nucleic acids from other components present in the amplifying composition; k) denaturing double-stranded nucleic acids;

l) separating the unbound single-stranded nucleic acids from nucleic acids bound to the solid support; and m) determining the mass of the single-stranded nucleic acids using mass spectrometry. A general schematic of the method is shown in Figure 1.

In other embodiments, the invention provides a method for identifying and/or quantitating at least one molecule or compound (sometimes referred to herein generally as an antigen) present in a sample suspected of containing the molecule(s) or compound(s), wherein the method comprises: a) providing at least one antibody (referred to hereafter as the "first" antibody) that specifically binds to a molecule or compound, wherein the first antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture; b) providing a sample that is suspected of containing a molecule or compound of interest; c) combining the first antibody and the sample to form a first assay composition; d) subjecting the first assay composition to conditions under which the first antibody and molecule or compound, if present, can specifically interact, e) combining a second antibody, which is tagged with a nucleic acid and which is specific for the molecule or compound of interest, with the first antibody, which will be bound to the analyte of interest if it was present in the tested sample, to form a second assay composition; f) subjecting the second assay composition to conditions under which the second antibody and molecule or compound, if present, can specifically interact, wherein specific interaction results in formation of a complex between the first antibody, the molecule or compound, and the second (tagged) antibody; g) separating the complex from other components present in the second assay mixture; h) amplifying the nucleic acid tag bound to the second antibody in an amplifying composition; i) binding amplified nucleic acid molecules to a solid support, wherein the solid support is different than the solid support linked to the first antibody; j) separating the bound amplified nucleic acids from other components present in the amplifying composition; k) modifying the nucleic acid tag; l) denaturing double-stranded nucleic acids; m) separating the unbound single-stranded nucleic acids from nucleic acids bound to the solid support; and m) determining the mass of the single-stranded nucleic acids using mass spectrometry.

In embodiments of the method described immediately above, nucleic acids in step k) can be modified in any suitable manner that permits subsequent analysis by mass spectrometry. In one example of this embodiment, the bound double-stranded tag is DNA, which can be cleaved by a restriction endonuclease. Cleavage is at a known, pre-determined or pre-engineered site, and cleavage releases a double-stranded fragment. The released fragment can be isolated from the bound tag, and the mass determined. Alternatively, after isolation away from the tag, the fragment can be denatured, and the mass of single-strands of the fragment determined.

In another example of this embodiment, the bound double-stranded tag is a DNA tag, which can be cleaved by a restriction endonuclease at a known, pre-determined or pre-engineered site, to release a double-stranded fragment. The DNA tag can then be purified away from the cleaved fragment. The DNA tag can then be further modified by nicking with a nicking enzyme, resulting in cleavage of the non-tagged strand of the DNA tag. The double-stranded DNA tag can then be denatured, releasing a single-stranded molecule having a pre-selected mass. The released single-stranded molecules can then be isolated from nucleic acids bound to the solid support. Then, the mass of the single-stranded nucleic acid can be determined using mass spectrometry. In embodiments, this technique is used to cleave off the nucleic acid sequences introduced via the PCR primers. Thus, this scheme effectively separates the design of the primers from the design of the mass tags. A general schematic of this example of this embodiment is shown in Figure 3.

The solid support that is linked to the first antibody is any physical component that is suitable for binding antibodies or antibody fragments or derivatives, reversibly or irreversibly. For example, it can be cellulose or a cellulose derivative, such as nitrocellulose; nylon or a nylon derivative; latex or a latex derivative; or any other material known to those of skill in the art as suitable for binding antibodies or proteins. In embodiments, the solid support is a particle having a surface to which at least one antibody is attached via hydrophobic, electrostatic, ionic, or covalently bonds, or by van der Waals forces. In embodiments, the solid support is a latex bead. In embodiments, it is a cellulose bead or particle. In embodiments, it is a magnetic bead, which can be, but is not necessarily, biotinylated. The particle can be solid or

porous, and the first antibody can be attached solely at the surface or throughout the volume of the particle, directly or via a linking group. In embodiments, the solid support is a membrane.

The sample suspected of containing the molecule or compound of interest can be any type of sample. That is, it can be in the form of a solid, liquid, or gas. If it is in the form of a solid or gas, techniques can be used to solubilize the sample in the assay solvent prior to, or during, practice of the method. For example, if it is in the form of a solid, it can be dissolved, dispersed, or otherwise suspended in an appropriate solvent, so that the molecule or compound, if present, will be available and capable of specifically interacting with the first antibody. If necessary, the materials in the sample can be concentrated or diluted prior to addition to the first antibody. Concentration or dilution might be necessary or desirable depending on the amount of material present in the sample, as obtained from the source.

In embodiments, the sample comprises biological material. By biological material, it is meant organic material that is produced through biological activity and that is used by a living organism for generation or maintenance of life. Examples include, but are not limited to, protein, polysaccharide, nucleic acid, fatty acid, lipid, and other macromolecules typically seen in biological cells. Examples also include, but are not limited to, metabolites or metabolic products of ingested or injected substances. The sample can contain a single species of biological material (*e.g.*, a purified protein), or a mixture of more than one species of biological material. The biological material can be of a known identity, or it can be of unknown identity. Samples containing known components can be used as internal controls to judge the accuracy, sensitivity, or reliability of the system and method of the invention. Accordingly, they can be used as standards against which other, unknown materials can be judged.

In embodiments, the sample is a bodily fluid from an animal, such as a mammal. In embodiments, the mammal is a dog. In embodiments, the mammal is a cat. In embodiments, the mammal is a horse. In embodiments, the mammal is a cow or other bovine. In embodiments, the mammal is a sheep. In embodiments, the mammal is a pig. In embodiments, the mammal is a human. In embodiments, the

sample is serum. In embodiments, the sample is urine. In embodiments, the sample is saliva.

In embodiments, the sample contains non-biological material, including organic and/or inorganic material. Non-biological organic material includes, but is not limited to, organic waste products that are not further metabolized by the organism that produces them. However, it should be noted that this definition does not exclude them being biological material for another organism. The organic non-biological material can be as small as methane or as large as long-chain fatty acids, lipids, or carbohydrates. Inorganic materials are any materials that do not comprise carbon as a component element. They include, but are not limited to, salts, metals, minerals, and the like. Other inorganic materials include, but are not limited to, metabolites and metabolic products.

Further examples of biological and non-biological materials that can be assayed using the present system and method are discussed below.

The method of the invention relies on specific antibody-antigen binding between the first antibody and the molecule or compound (the antigen) and between the second antibody and the molecule or compound in assay compositions. Conditions (and thus compositions) under which specific antibody-antigen binding can occur are known to those of skill in the art or can be determined using standard, routine techniques which do not require undue or excessive experimentation. Accordingly, they need not be specifically disclosed herein. Furthermore, the amounts (total number of molecules or total volume) of each antibody that is added can be adjusted based on the amounts of sample and reagents added to perform the method. Such adjustments can be made without undue or excessive experimentation.

Once the first antibody and molecule(s) or compound(s) have been exposed to each other for a sufficient amount of time under conditions such that specific binding of the first antibody and the molecule(s) or compound(s), if present, has taken place, the first antibody, which is bound to the solid support, can be separated from unbound molecules and compounds present in the first assay composition, and thus from the composition itself. The first antibody/solid support can be, but is not necessarily, resuspended in an appropriate solvent (*e.g.*, assay or binding buffer). Likewise, it can

be, but is not necessarily, added directly to a composition comprising a second, tagged antibody, as discussed below. At any time, portions of the compositions or materials can be removed for later use, for example, to assay reaction efficiencies, etc.

The first antibody/solid support (with or without bound molecule/compound) can be removed from other materials present in the first assay composition by any suitable method, including centrifugation to pellet the antibody/solid support, magnetic separation by application of a magnetic field to attract magnetic beads, or direct, physical removal of the solid support from the assay composition (*e.g.*, physically lifting a membrane from the assay composition). When desired, the antibody/solid support can be washed one or more times with an appropriate solvent or solvent composition (*e.g.*, a wash buffer) to further remove unbound (or non-specifically bound) molecules or compounds from the antibody/solid matrix. Techniques for washing antibody/solid support complexes are known to those of skill in the art, and any suitable technique can be used.

Likewise, once the labeled second antibody has been exposed to the first antibody/solid support, which might or might not be bound to an antigen of interest, under conditions sufficient to permit specific binding of the second antibody to solid support/first antibody/antigen complexes, the complexes are separated from unbound materials and optionally washed one or more times to remove unbound or non-specifically bound materials.

The second antibody is tagged with a nucleic acid of known length and base composition. In embodiments where multiple molecules or compounds are to be detected, multiple second antibodies, each specific for a single molecule or compound of interest, are used. Each specific second antibody will contain a nucleic acid tag that is different, or can be rendered different by modification, from the tag of every other second antibody to be used. In this way, the method provides sufficient different tags to detect each molecule or compound of interest.

The nucleic acid tags may be of any length or composition. The size and composition can depend on the number of molecules or compounds that are to be assayed. The size and composition should be selected in conjunction with the parameters to be used for mass-spectrometry detection of the labels. Any type of

nucleic acid can be used, such as double-stranded DNA, single-stranded DNA, double-stranded RNA, single-stranded RNA, or hybrids of RNA and DNA strands. In embodiments, the tags have pre-determined primer binding sites to facilitate amplification of the tag. The primer binding sites can be of any length and base sequence. Primer design, and in particular design of primer pairs for amplification of double-stranded nucleic acids, is well within the abilities of those of skill in the art and need not be detailed herein. Where the tag is to be modified prior to mass spectrometry analysis, suitable sites for the modification (*e.g.*, restriction cleavage, nicking) should be included in the tag.

When a molecule or compound (antigen) of interest is present in the sample, it will specifically bind to the first antibody. Upon addition of the tagged second antibody, the second antibody will specifically bind to the solid support/first antibody/antigen complex, forming a solid support/first antibody/antigen/tagged second antibody complex. After removal of unbound and, optionally, non-specifically bound material, the nucleic acid tag is then amplified to increase the number of copies of each tag present in the complex. Amplification can be by any suitable means. In embodiments, amplification is performed by the polymerase chain reaction (PCR). Techniques for performing PCR are well known to those of skill in the art and need not be detailed here. The nucleic acid tags can be amplified to the extent desired by the practitioner. The amplified nucleic acids will be double-stranded at this point in the procedure.

In embodiments, one or both of the primers used for amplifying the tags are themselves labeled or otherwise modified so that they can be selectively bound to a second solid support. In embodiments where both primers are labeled, either one or two second solid supports can be used. When two solid supports are used in conjunction with two labeled primers, each support will specifically bind only one of the primers. Use of two labeled primers and two solid supports permits additional flexibility in the practice of the invention and also permits the practitioner to determine the relative efficiency of various labels/solid supports.

In order to ensure that the amplified tags are adequately detected, the labels used to label the primers should not specifically, or to any significant extent, bind to



the solid support used to bind the first antibody. Suitable labels for the primers include, but are not limited to, biotin and antibodies or antibody fragments.

After amplification, the amplified nucleic acid molecules are bound to a solid support through the labels incorporated into the primer(s). The bound amplified nucleic acids are then separated from other components present in the amplifying composition (for example, unincorporated primers and nucleotides). Techniques for separating the nucleic acid/solid support are well known and need not be detailed here. If desired, the separated nucleic acid/solid support can be washed to further remove unbound or non-specifically bound materials using standard techniques.

Once the double-stranded amplified tags are separated from other materials, they are denatured using any suitable technique. Suitable techniques include, but are not limited to, heat denaturation and chemical denaturation (*e.g.*, pH or urea).

The single-stranded nucleic acids that are not bound to the solid support are then separated from the solid support/bound nucleic acids using suitable techniques. Optionally, the unbound nucleic acids are washed to remove unwanted materials.

Finally, the unbound single-stranded nucleic acids are detected and identified using mass spectrometry. As discussed above, each nucleic acid tag used will have a predetermined composition and weight (mass). Thus, detection of the mass of the tag by mass spectrometry will immediately identify the identity of the tag. Because the specificity of the antibody attached to each nucleic acid tag is known, the identity of the molecule(s) or compound(s) in the sample can be determined.

As an example of an application of the present invention, Figure 2a shows a histogram of masses for 10-base single-stranded DNA. Figure 2b shows that unique mass tags are spaced every ~1 Da (singly-charged ions) over a broad total range, so that a single-stranded DNA of 10 bases offers some 300 or more tags. Detection of these tags would require a mass accuracy of only 0.03% (300 ppm) and a resolving power of 3000 or so, both figures of merit well within the capabilities of modern MALDI-MS instrumentation (ref. 24). To avoid mass spectral overlap of isotopic distributions associated with mass tags that are too close in mass, it might be desirable to space the tags every  $\delta(m/z) \sim 5$ . This would still give about 60 mass tags, all composed of just 10 bases. Other spacings may be used, and can be selected based on

the number of tags to be used and the masses of the tags. Use of longer nucleotide sequences would increase the number of potential tags. For a single-stranded DNA of 15 bases, 120 to 600 mass tags can be obtained. For a single-stranded DNA of 20 bases, 160 to 800 mass tags can be obtained. The range obtainable depends, at least in part, on the smallest spacing between adjacent pairs. Current MALDI-MS instruments can distinguish anywhere from about a 1 Dalton difference to about a 5 Dalton difference in spacing.

Therefore, by judicious choice of nucleic acid sequences, one can design hundreds to thousands of unique tags for various desired analytes. For example, Figure 2 shows histograms of  $m/z$  values of singly-charged DNA. The plots show histograms centered around  $m/z \sim 3100$ , and show that use of 10 bases can provide a substantial plurality of DNA mass tags that can easily be resolved by MS.

It should be apparent from the disclosure that, since all of the antibodies, tags, primers, and solid supports will be added in known amounts, the present method can not only detect, indirectly, the presence of antigens of interest, but it can provide information on the quantity of the antigens in the original sample as well. Thus, the present invention provides not only a qualitative method for detection and/or identification of molecules and compound, but a quantitative or semi-quantitative method for detection and/or identification.

The method of the invention is suitable for practice by humans manually. It is also suitable for automation, using multi-well formats, fluidic robotics, and chip technology.

The method is capable of detecting any material that is capable of being detected by antibodies (including antibody fragments and other molecules derived from antibodies). Included among the materials that can be detected are living organisms or cells, such as prokaryotes and eukaryotes. Examples of prokaryotic organisms or cells include, but are not limited to, gram positive and gram negative bacteria associated with animal (including human) disease, as well as environmental bacteria associated with plant or animal disease. Examples of eukaryotic organisms or cells include, but are not limited to fungi, protozoa, and cancer cells. Detection of a given organism or cell can be based on binding of an antibody known to be specific

for that organism or cell, or binding of a panel of antibodies, where the binding pattern of the panel is typical of a given organism or cell. Binding can be to any portion of the organism or cell, as discussed below.

Entities not widely considered as living, but having properties of living entities can be detected as well. As an example, prions, such as those implicated in bovine spongiform encephalopathy (BSE, or "mad cow disease") and Creutzfeld-Jacob disease, can be detected with the present method.

One of the many materials that can be detected is protein. There is no limit to the type and source of protein that can be detected. Exemplary proteins include structural proteins such as enzymes, cell structure components, and receptors and other cell membrane, cell surface, or viral coat proteins. Other examples include regulatory proteins of prokaryotes, eukaryotes, and viruses, such as proteins that affect the level of transcription of nucleic acids, and proteins that post-transcriptionally or post-translationally modify nucleic acids or proteins. Further exemplary proteins include modified proteins such as glycoproteins, phosphoproteins, and other proteins with post-translational modifications. Naturally made, recombinant, and chemically synthesized proteins can likewise be detected with the systems and methods of the invention. Included within proteins are protein fragments, polypeptides, and peptides.

In embodiments, the method of the invention is used to detect and differentiate between a single protein that is capable of existing and functioning with more than one three-dimensional conformation. It is known that many proteins have different activities, or different levels of activities, based on their secondary or tertiary structures. For example, a protein might be inactive in its native, translated state, but become activated upon phosphorylation, which results in a change in its three-dimensional conformation. Examples of proteins that are conformationally activated/inactivated by post-translational modifications include those that are cell-cycle dependent, those that are involved in sensing and transducing signals from the outside of the cell to the inside, those that are involved in senescence/quiescence, and those involved in infection or evasion of the immune system. As discussed further herein, antibodies have been, and will continue to be, developed that identify one configuration of a protein, but not another. Accordingly, because the method of the

invention can identify different conformations of a protein, and because certain proteins have different conformations depending on their cell cycle and/or environment, the present method can be used to identify the metabolic and/or cell cycle state of an organism or cell of interest.

Another of the many materials that can be detected using the method of the invention are relatively small, bioactive compounds, such as drugs or pharmaceuticals. As used herein, a drug is any compound or molecule that has a biological activity when exposed to an animal, including a human. A pharmaceutical is a drug that has a therapeutic effect when exposed to an animal, including a human. Thus, a pharmaceutical is a drug, but a drug is not necessarily a pharmaceutical. While not all drugs or pharmaceuticals can be detected with antibodies, some can. Furthermore, while drugs or pharmaceuticals themselves are often undetectable with antibodies, the effects of the drugs or pharmaceuticals on the cells of an individual exposed to those drugs or pharmaceuticals can often be detected. Thus, in embodiments, the method is a method of detecting a drug in a sample. In embodiments, the method is a method of detecting a pharmaceutical in a sample.

Other applications of the system and method of the invention include:

Environmental Sensing. As our world increases in complexity, there is a growing need to monitor the presence of any number of materials or substances in an environmental context, including both natural and man-made environments. Natural environments in which it is necessary to trace various substances include ground water, lakes, rivers, water runoff, and the atmosphere (which can deliver harmful airborne substances). Man-made environments of significance include, but are not limited to: home, occupational, transportation, and recreational. Note that natural and man-made environments are connected, with materials and substances flowing between them. Bioenvironmental sensing/monitoring is one way to understand these flow processes and to improve our quality of life. With enhanced sensing capabilities provided by the present invention, a more complete picture of what harmful substances are in the environment will be obtained, and better knowledge will be had on how these substances flow through the environment. The need to monitor covers both inorganic and organic small molecules, *e.g.*, pollutants, pesticides, poisons, and

industrial byproducts; as well as living organisms, like cells, viruses, bacteria, and associated molecules of all types. With parallel sensing of enhanced nature, as provided by the invention, trends which are now barely perceived will become clear.

Medical Applications. It is here where a great benefit of the invention is achieved. There are many kinds of molecules of medical importance that can be detected immunologically, and that can be analyzed in parallel by the present invention. Examples include, but are not limited to, drugs, metabolites, hormones, cells, cell markers, and tumor markers.

Considering tumor markers, it is known that under certain circumstances, cancer cells excrete or present specific molecules which normal cells do not. Antibodies specifically raised against such marker molecules, and deployed in parallel as in the present invention, can provide an effective means of assaying a variety of cancers simultaneously. Pre-cure, the method can be used to identify cancer cells. It can be used to monitor and localize the spread of cancer as well. Post-cure, the method can be used to monitor or detect recurrence of the cancer. It should be noted that at present, when it is believed that a certain molecule is secreted by an abnormal cell but not a healthy one, it might actually be that the healthy cell emits the molecule too – but in quantities too small to be observed with present technology. Using the present invention, which provides greater sensitivity than the immuno-PCR techniques currently available, it can be determined whether the detected molecule is also secreted by normal cells. Such an observation would shed light on the distinction between normality and abnormality and aid in devising cures for diseases.

It is known that bacteria secrete characteristic substances. In the food industry, the present invention can be used to monitor the hygiene of food products by scanning for the products (*i.e.*, signatures) of various bacteria.

It should be noted that certain molecules of analytical importance are present in living organisms (such as humans) in amounts too small to measure by currently available techniques. Among these molecules are important proteins that might be present in cells at very low levels, for example only a few copies per cell. The present invention enables one to identify such molecules, which not only permits a better profile of the cell to be generated, under normal and abnormal conditions, but also

provides information on "metabolomes", that is, the full complement of metabolites present in a cell or cell population at a given time. Further, it provides information on collections of proteins and cell macromolecules that are coordinately expressed or produced under various environmental conditions.

The present invention provides, for example, a single assay to test for the presence of a variety of illicit drugs in the human body (and in racing horses, dogs, *etc.*) in trace amounts. Likewise, it allows one to follow the fate of a wide array of so-called biological "markers", such as molecules that serve to highlight the metabolic fate of ingested substances. Such an application bridges the applicability of this invention between environmental and health issues.

With the enhanced parallel sensing of the invention, trends which are now barely perceived will become clear. Thus, in some cases, the invention provides an advanced warning system, such as for emergence of new infectious strains of viruses and bacteria.

Forensics. The present invention aids in solving any crime in which establishing the presence or absence of certain trace substances is of importance and in which presence or absence of the substances can be established by immunological methods.

Phenotype Profiling. A major goal in post-genomic research is to provide sensitive, selective, rapid, and parallel monitoring of a multitude of proteins, metabolites, drugs, *etc.* as a function of the state of an organism. With proteins, even the status of post-translational modifications has significant functional implications. It is considered imperative to seek improvements to existing proteomics technology, which are based to a large extent on two-dimensional gels. Unfortunately, these techniques suffer from lack of sophistication and require multiple, laborious and often tedious interactions from human laborers. The present invention, which utilizes immunological techniques coupled to nucleic amplification and mass spectrometry, provides a new method for assaying proteomes and metabolomes, which comprise a plurality of diverse targets. As discussed above, antibodies have been, and can further be, raised against proteins, peptides, metabolites, and even inorganic particles. Furthermore, they can be raised to be sensitive to protein conformation and to the state

of post-translational modification. The construction of small libraries of useful probe antibodies can be accomplished by those of skill in the art using routine techniques. The construction of large libraries has begun and such libraries will be widely available in the near future. Therefore, the present invention is useful for phenotype profiling, which in turn has ever increasing applications in medicine.

In another aspect, the invention provides a kit for practicing the method of the invention. The kit comprises some or all of the reagents and supplies for practicing the method of the invention, packaged separately or in combination in a suitable container. Containers according to the invention can be, but are not necessarily, capable of being sealed, such that they are, either minimally, substantially, or totally, gas and/or liquid impermeable. In embodiments, the containers are sterile prior to addition of the components according to the invention (*e.g.*, antibodies, assay buffers, wash buffers).

Kits according to the invention contain some or all of the reagents and supplies for practicing the method of the invention. In embodiments, the kits provide at least one labeled second antibody according to the invention. As discussed above, the labeled second antibody is labeled with a nucleic acid of known length and base composition. The second antibody also has a known specificity for antigen. In embodiments, more than one labeled second antibody are provided, either in a single container or in separate containers containing only one type of antibody. In embodiments, combinations of labeled second antibodies are provided in one or more containers within the kit. In embodiments, two or more labeled second antibodies are provided in the kit. In embodiments, ten or more are provided. In embodiments, forty or more are provided. In embodiments, one hundred or more are provided.

The kits may also contain at least one first antibody, either bound to a solid support, or not. In embodiments where the first antibody is not provided bound to the solid support, the kit may further comprise the solid support in another container. In embodiments one first antibody is provided. In embodiments, combinations of more than one first antibodies are provided in one or more containers within the kit. In embodiments, two or more first antibodies are provided in the kit. In embodiments,

ten or more are provided. In embodiments, forty or more are provided. In embodiments, one hundred or more are provided.

In accordance with the invention, analytical kits keyed to a wide variety of harmful substances likely to be present in certain types of environments are provided. For example, a kit for monitoring for a variety of river pollutants is provided. Likewise, a kit for monitoring for pollutants characteristic of a mining process plant is provided. Other kits according to the invention include, but are not limited to, kits for detecting bacteria, kits for detecting viruses, kits for detecting cancer cells, and kits for detecting prions. For example, in embodiments, the invention provides kits that are designed to monitor for antigens, or sets of antigens, that are specific for a disease or an organism that is a causative agent of a disease.

2



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**What is claimed is:**

1. A method for detecting the presence of an antigen in a sample suspected of containing the antigen, said method comprising:

- a) providing at least one first antibody that specifically binds to an antigen, wherein the first antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture;
- b) providing a sample that is suspected of containing the antigen;
- c) combining the first antibody and the sample;
- d) combining a second antibody, which is tagged with a nucleic acid and which is specific for the antigen, with the first antibody to form a second assay composition;
- e) separating any complexes of first antibody/antigen/second antibody from other components present in the second assay composition;
- f) amplifying the nucleic acid tag bound to the second antibody; and
- g) detecting the presence of amplified nucleic acid by mass spectrometry, wherein the presence of amplified nucleic acid indicates the presence of the antigen in the original sample.

2. The method of claim 1, further comprising determining the amount of amplified nucleic acid.

3. The method of claim 1, wherein the antigen is a protein.

4. The method of claim 1, wherein the antigen is a drug or pharmaceutical.

5. The method of claim 1, wherein the antigen is a protein and the method detects the conformational status of the antigen.

6. The method of claim 1, wherein the antigen is a protein and the method detects the cell cycle status of the cell from which the protein came.

7. The method of claim 1, wherein the antigen is a protein and the method detects the metabolic status of the cell from which the protein came.
8. The method of claim 1, wherein the sample is a biological sample from a human and the method detects the presence of cancer cells in the human.
9. The method of claim 1, wherein the sample is a biological sample from a human and the method detects the spread or recurrence of cancer cells in the human.
10. The method of claim 1, wherein the method is automated.
11. The method of claim 1, wherein the sample is from a human.
12. The method of claim 1, wherein the sample is serum.
13. The method of claim 1, wherein the sample is urine.
14. A method for detecting the presence of an antigen in a sample suspected of containing the antigen, said method comprising:
  - a) providing at least one first antibody that specifically binds to an antigen, wherein the first antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture;
  - b) providing a sample that is suspected of containing the antigen;
  - c) combining the first antibody and the sample to form a first assay composition;
  - d) subjecting the first assay composition to conditions under which the first antibody and antigen, if present, can specifically interact;
  - e) combining a second antibody, which is tagged with a nucleic acid and which is specific for the antigen, with the first antibody, to form a second assay composition;

f) subjecting the second assay composition to conditions under which the second antibody and antigen, if present, can specifically interact, wherein specific interaction results in formation of a complex between the first antibody, the antigen, and the second antibody;

g) separating the complex from other components present in the second assay mixture;

h) amplifying the nucleic acid tag bound to the second antibody;

i) binding amplified nucleic acid molecules to a solid support, wherein the solid support is different than the solid support linked to the first antibody;

j) separating the bound amplified nucleic acids from other components present in the amplifying composition;

k) denaturing double-stranded nucleic acids;

l) separating the unbound single-stranded nucleic acids from nucleic acids bound to the solid support; and

m) determining the mass of the single-stranded nucleic acids using mass spectrometry.

15. The method of claim 14, further comprising determining the amount of amplified nucleic acid.

16. The method of claim 14, wherein the antigen is a protein.

17. The method of claim 14, wherein the antigen is a drug or pharmaceutical.

18. The method of claim 14, wherein the antigen is a protein and the method detects the conformational status of the antigen.

19. The method of claim 14, wherein the antigen is a protein and the method detects the cell cycle status of the cell from which the protein came.

20. The method of claim 14, wherein the antigen is a protein and the method detects the metabolic status of the cell from which the protein came.
21. The method of claim 14, wherein the sample is a biological sample from a human and the method detects the presence of cancer cells in the human.
22. The method of claim 14, wherein the sample is a biological sample from a human and the method detects the spread or recurrence of cancer cells in the human.
23. The method of claim 14, wherein the method is automated.
24. The method of claim 14, wherein the sample is from a human.
25. The method of claim 14, wherein the sample is serum.
26. The method of claim 14, wherein the sample is urine.
27. A method for detecting the presence of an antigen in a sample suspected of containing the antigen, said method comprising:
  - a) providing at least one first antibody that specifically binds to an antigen, wherein the first antibody is linked to a solid support or some other physical component that can be selectively removed or partitioned from an assay mixture;
  - b) providing a sample that is suspected of containing the antigen;
  - c) combining the first antibody and the sample to form a first assay composition;
  - d) subjecting the first assay composition to conditions under which the first antibody and antigen, if present, can specifically interact;
  - e) combining a second antibody, which is tagged with a nucleic acid and which is specific for the antigen, with the first antibody, to form a second assay composition;

f) subjecting the second assay composition to conditions under which the second antibody and antigen, if present, can specifically interact, wherein specific interaction results in formation of a complex between the first antibody, the antigen, and the second antibody;

g) separating the complex from other components present in the second assay mixture;

h) amplifying the nucleic acid tag bound to the second antibody;

i) binding amplified nucleic acid molecules to a solid support, wherein the solid support is different than the solid support linked to the first antibody;

j) optionally, cleaving the nucleic acid tag with a restriction endonuclease;

k) optionally, nicking one strand of the amplified nucleic acid tag;

l) denaturing the nucleic acid tag;

m) separating the unbound single-stranded nucleic acids from nucleic acids bound to the solid support; and

n) determining the mass of the single-stranded nucleic acids using mass spectrometry,

wherein one or both of steps j) and k) are performed.

28. The method of claim 27, further comprising determining the amount of amplified nucleic acid.

29. The method of claim 27, wherein the antigen is a protein.

30. The method of claim 27, wherein the antigen is a drug or pharmaceutical.

31. The method of claim 27, wherein the antigen is a protein and the method detects the conformational status of the antigen.

32. The method of claim 27, wherein the antigen is a protein and the method detects the cell cycle status of the cell from which the protein came.



33. The method of claim 27, wherein the antigen is a protein and the method detects the metabolic status of the cell from which the protein came.
34. The method of claim 27, wherein the sample is a biological sample from a human and the method detects the presence of cancer cells in the human.
35. The method of claim 27, wherein the sample is a biological sample from a human and the method detects the spread or recurrence of cancer cells in the human.
36. The method of claim 27, wherein the method is automated.
37. The method of claim 27, wherein the sample is from a human.
38. The method of claim 27, wherein the sample is serum.
39. The method of claim 27, wherein the sample is urine.

1/3

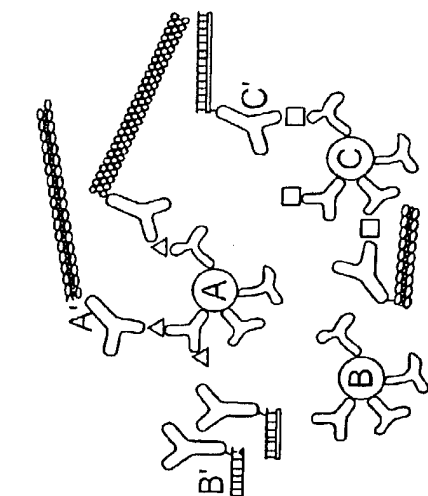


FIG. 1c

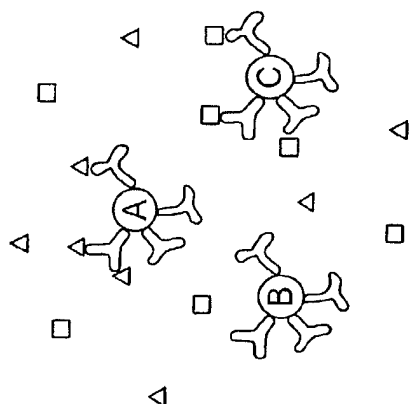


FIG. 1b

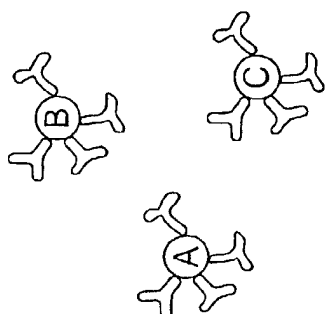


FIG. 1a

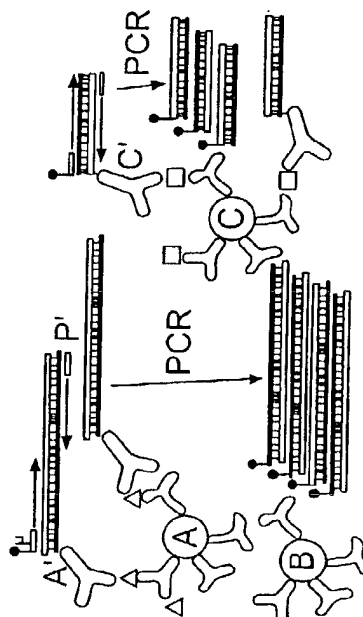


FIG. 1d

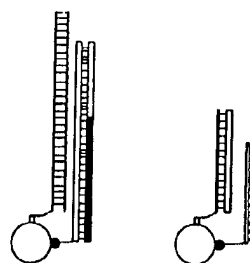


FIG. 1e

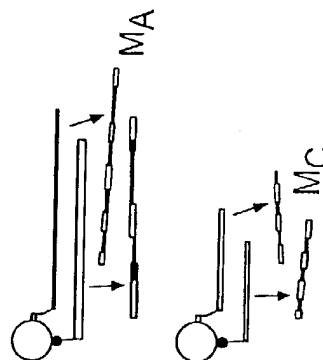
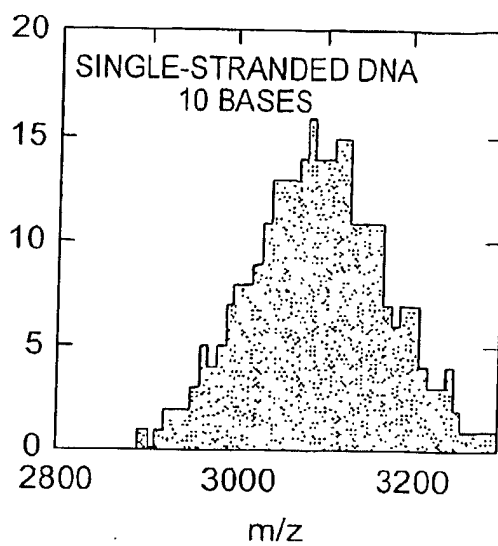
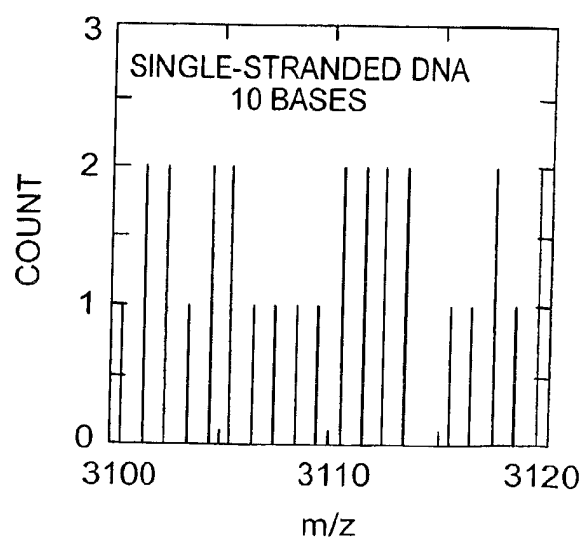


FIG. 1f

2/3

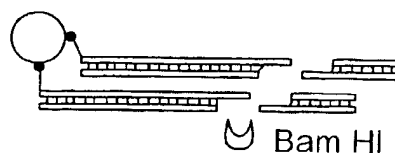


**FIG. 2a**

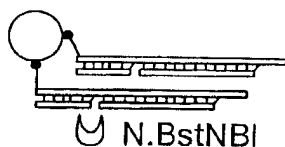


**FIG. 2b**

3/3



Bam HI



N.BstNBI

**FIG. 3a**

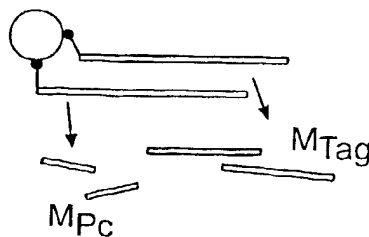
BamHI RESTRICTION SITE:

...G | GATCC...  
...CCCTAG | G...

N.BstNBI NICKING SITE:

...GAGTCNNNN|...  
...CTCAGNNNN....

**FIG. 3b**



**FIG. 3c**

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WO 01/84146 A3

(54) Title: A SENSITIVE, MULTIPLEXED MASS-SPECTROMETRY-BASED BIOSENSOR BASED ON IMMUNO-PCR

(57) Abstract: The invention provides a method for detection of substances. The method utilizes immunological detection of a substance, followed by amplification of a nucleic acid tag bound to the antibody, and identification of the amplified tag. The detection method utilizes mass spectrometry to detect specific binding of the antibody to an antigen by detecting the presence, and identifying the mass, of the nucleic acid tag.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 01/00957

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N33/53 C12Q1/68 H01J49/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q H01J C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 29432 A (UNIV BOSTON) 26 September 1996 (1996-09-26) page 12, line 19 - line 25; claims 1-4,22 ---	1-13
X	WO 99 38013 A (XERION PHARMACEUTICALS GMBH) ) 29 July 1999 (1999-07-29) page 23 -page 24; claims 1-6 ---	1-13
P,A	WO 00 57183 A (BIOVATION LTD ) 28 September 2000 (2000-09-28) abstract; claims 1-24 --- -/--	1-39

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"B" document member of the same patent family

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 01/00957

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARIAN C CASE ET AL: "Enhanced ultrasensitive detection of structurally diverse antigens using a single immuno-PCR assay protocol" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 223, 1999, pages 93-106, XP002901935 the whole document ---	1-39
A	DATABASE BIOSIS, PREV199800180103 [Online] Genetic Analysis Biomolecular Engineering, January 1998 (1998-01) JURINKE CHRISTINA ET AL: "Application of nested PCR and mass spectrometry for DNA-based virus detection: HBV-DNA detected in the majority of isolated anti-HBc positive sera" XP002901936 abstract -----	1-39

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Information on patent family members

International Application No

PCT, IB 01/00957

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			WO	9629432 A1	26-09-1996
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			WO	0057183 A1	28-09-2000